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Characterization and milk coagulating properties of *Cynanchum otophyllum* Schneid. proteases

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ABSTRACT

The herbaceous plant, *Cynanchum otophyllum* Schneid., is widely used as a milk coagulant to make a Chinese traditional milk product, milk cake. However, the milk-clotting compounds and their mechanism remain unclear. In this study, crude proteases were extracted from the dried leaves of *Cynanchum otophyllum* Schneid. using citric acid-phosphate buffer and then partially purified by weak anion exchange chromatography. Two proteases, QA and QC, with molecular weights of 14 and 27 kDa, respectively, were shown to exhibit milk-clotting activity. A study of the effects of pH and temperature on the milk-clotting activity and proteolytic activity of the proteases showed that they exhibited good pH stability from pH 5.5 to 7.5 and good thermal stability at temperatures from 50 to 70°C. The QA and QC were the cysteine proteases, able to hydrolyze β-casein and κ-casein completely, and α-casein partially. The cleavage site on κ-casein determined by Orbitrap (Thermo Fisher Scientific, San Jose, CA) analysis showed that QA and QC could cleave κ-casein at Ser132-Thr133. Overall, the results suggest that the *Cynanchum otophyllum* Schneid. proteases are a promising milk-clotting enzyme that could be used for manufacturing milk cake and cheese.
Key words: *Cynanchum otophyllum* Schneid., milk-clotting activity, proteolytic activity, plant rennet

INTRODUCTION

Milk cake is a traditional milk product which has been consumed for over 600 yr by people from the minority ethnic region in Yunnan province, China (Xiao et al., 2007). Milk cake is known as Chinese cheese and is usually made of goat, cow, or buffalo milk. Because of its high nutritional value, special flavors, and unique taste, milk cake has become the favorite food of the local people of Yunnan (Tao et al., 2015). However, milk cake is mostly produced by hand in workshops, leading to low yields and uncertain quality, so its manufacturing processes need to be improved.

As milk cake is a type of ready-to-eat fresh cheese, milk clotting directly affects its yield, texture, and sensory quality. Traditionally, milk cake is made typically by heating milk in combination with acid and rennet gelation. Generally, after heating the raw milk to boiling point, acid whey is added with or without coagulant, then the curd is compressed and molded (Xiao et al., 2007). People in the JianChuan and HeQing counties of Yunnan province have accidentally found that the solution produced by soaking a local plant, *Cynanchum otophyllum* Schneid., could be used as a coagulant to make milk cake. However, manufacturing this solution has not been standardized regarding the soaking temperature and ratio of water to leaves, so that the milk-clotting activity (**MCA**) of the solution can differ greatly. Therefore, developing a coagulant to meet the demands of industrial production of milk cake is very important.

Cynanchum otophyllum Schneid., which belongs to the *Asclepiadacene* family, is a perennial grass vine that grows in the mountains or valley woodlands at elevations of 1,500 to 2,800 m, particularly in Jianchuan county, Yunnan province (Shen et al., 2014). Studies on *C. otophyllum* have focused mainly on its pharmacological effects, with its active ingredients being identified as steroid esters that have important biological functions such as anti-epileptic effects (Zhao et al., 2013). However, the milk-clotting mechanism of *C. otophyllum* remains unstudied.

Calf rennet, which consists of over 90% chymosin, has been widely used in the manufacture of cheese (Kumar

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et al., 2010). The global increase in cheese production coupled with the preferences of religious and vegetarian consumers has prompted a search for an alternative milk-clotting coagulant from a nonanimal source, such as plant rennet. Several proteases extracted from plants such as kiwi, papaya, ginger rhizome, and cardoon (*Cynara cardunculus*) have been successfully used as milk-clotting coagulants for cheese making in different parts of the world (Jacob et al., 2011). Different plant extract proteases have also been shown to have particular active ingredients, cleavage sites, and conditions for optimal application (Chen et al., 2003; Vairo-Cavalli et al., 2005; Brutti et al., 2012). *Cynanchum otophyllum* is a promising plant rennet so separating and purifying its proteases and clarifying its milk-clotting mechanism are very important and need to be investigated.

The present study aims to separate and purify the proteases of *C. otophyllum,* then to determine the effects of temperature and pH on the MCA and proteolytic activity (**PA**) of the proteases. The products of protease degradation and the cleavage sites of the enzymes on κ-CN will also be investigated. A better understanding of the milk-clotting characteristics of the *C. otophyllum* proteases could contribute to developing a plant rennet for the industrial production of milk cake.

MATERIALS AND METHODS

Samples and Reagents

Samples of *C. otophyllum* Schneid. (Chinese name Qingyangshen) were collected from Jianchuan county (2,000 m above sea level) in Dali City, Yunnan province. After natural drying, the *C. otophyllum* samples were stored in a freezer at −20°C until processing. Skim milk powder was obtained from Nouriz Dairy Co. (Shanghai, China); Q Sepharose Fast Flow from GE Healthcare (Uppsala, Sweden); calf rennet, Naturen Stamix 1150 NB, from Chr. Hansen (Hoersholm, Denmark); bromelain, papain, whole casein, α-CN, β-CN, κ-CN, and BSA from Sigma-Aldrich (St. Louis, MO); Coomassie Brilliant Blue G-250 and R-250 from Bio-Rad Laboratories (Hercules, CA); and 10-kDa ultrafiltration tubes from Millipore (Billerica, MA). All other chemicals were of analytical grade.

Extraction and Partial Purification of Protease

Extraction. The *C. otophyllum* proteases were extracted as described by Huang et al. (2011) with some modifications. The *C. otophyllum* leaves were cut into pieces, mixed with 10 mmol/L of citric acid-phosphate buffer (pH 6.5, containing 1.0 mmol/L of EDTA and cysteine), then left for 2 h at 4°C.

Separation and Partial Purification. After successive filtration through 4 layers of cheesecloth and a 0.22-μm membrane, the filtrate was concentrated by ultrafiltration with a 10-kDa molecular weight cut-off membrane. The retentate was then applied to a E-C Polypropylene column (1.5 × 12 cm) packed with Q Sepharose Fast Flow, which had been equilibrated with 10 mmol/L of citric acid-phosphate buffer (pH 6.5). Elution was performed with gradient elute of 0, 0.6, and 1.0 mmol/L of NaCl (in binding buffer) to obtain 4 fractions of partially purified enzyme extracts. All fractions were monitored at 280 nm using a UV detector (UV-2102 PC, Unico Instrument Co. Ltd., Shanghai, China) to detect the proteins. The protein concentration of each fraction was measured following the method of Bradford (1976), whereas the molecular weights of the fractions were determined using SDS-PAGE as described by Laemmli (1970). All the extraction and purification processes were carried out below 10°C to protect the enzyme activity.

Milk Clotting Activity Assay

The MCA of the *C. otophyllum* proteases was determined using a modified method of He et al. (2011). One milliliter of substrate (12% skim milk in 10 m*M* $CaCl_2$, pH 6.5) was incubated at 37°C for 5 min, then 0.1 mL of enzyme was added. The time needed for curd formation was recorded and the MCA was expressed in Soxhlet units (**SU**). One SU of MCA was defined as the amount of enzyme required to clot 1 mL of substrate within 40 min at 37°C. The MCA of calf rennet, bromelain, and papain were also determined as a comparison.

Caseinolytic Activity Assay

The PA of the *C. otophyllum* proteases was determined using a method modified from Mohanty et al. (2003). The substrate was prepared by dissolving 1% (wt/vol) of whole casein in 10 mmol/L of citric acid-phosphate buffer (pH 6.5). The assay was performed by incubating 1.1 mL of substrate with 0.1 mL of partially purified enzyme at 37°C for 30 min and terminated using 1.8 mL of 5% (wt/vol) trichloroacetic acid (**TCA**). The control was prepared by adding the same amount of TCA to the protease, then adding the substrate. After 30 min standing at room temperature, the completely precipitated proteins were removed by centrifuging at 5,000 × *g* for 20 min at room temperature. The protein content in the supernatant was then measured at 280 nm (UV-2102 PC, Unico Instrument Co. Ltd.). One unit of PA was defined as the amount of enzyme extract required for an increase of 0.01 in optical density in 1 min at 280 nm.